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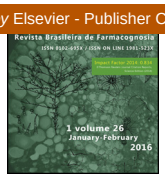

Original Article

# Chemical profiles of traditional preparations of four South American *Passiflora* species by chromatographic and capillary electrophoretic techniques


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ABSTRACT

Several species of the genus *Passiflora* are distributed all over South America, and many of these species are used in popular medicine, mainly as sedatives and tranquilizers. This study analyzes the chemical profile of extracts of four *Passiflora* species used in folk medicine, focusing on the flavonoids, alkaloids and saponins. We employed simple and fast fingerprint analysis methods by high performance liquid chromatography, ultra performance liquid chromatography and capillary electrophoresis techniques. The analysis led to the detection and identification of *C*-glycosylflavonoids in all the plant extracts, these being the main constituents in *P. tripartita* var. *mollissima* and *P. bogotensis*. Saponins were observed only in *P. alata* and *P. quadrangularis*, while harmane alkaloids were not detected in any of the analyzed extracts in concentrations higher than 0.0187 ppm, the detection limit determined for the UPLC method.



## Introduction

The genus *Passiflora* is the largest and most important genus of the family Passifloraceae, comprising about 500 species (Lewis and Elvin-Lewis, 1977). In North America and Europe, the main species, *P. incarnata*, is popularly known as passion fruit or passion-flower, while in South America, several others species of *Passiflora* are widely distributed and known by distinct names, such as 'maracujá', 'curuba', or 'badea', among others. (Arbelaez, 1996; Mors et al., 2000) Many of these species (*P. edulis* var. *edulis*, *P. edulis* var. *flavicarpa*, *P. tripartita* var. *mollissima* and others) are cultivated for their edible fruits or for the preparation of juices. Infusions of their leaves are also used in popular medicine in many countries, as a sedative or tranquillizer (Pio Corrêa, 1978; Arbelaez, 1996).

Different countries in South America have registered pharmaceutical preparations that use *Passiflora* species as the active component. In Colombia, for example, the leaves of *P. tripartita* var. *mollissima* are accepted as sedative and hypnotic component in phytopharmaceutical preparations (Invima, 2006). In Brazil, *P. alata* and *P. edulis* are included in the most recent version of the Brazilian Pharmacopeia (Farmacopeia Brasileira, 2010).

Regarding their chemical composition, the compounds more frequently reported for the genus are flavonoids, especially *C*-glycosylflavonoids, which are usually described as the main components (Ulubelen et al., 1982; Li et al., 2011; Zucolotto et al., 2012). These compounds have recently been associated with several pharmacological effects observed for distinct *Passiflora* species (Coleta et al., 2006; Santos et al., 2006; Sena et al., 2009; Zucolotto et al., 2009; Gazola et al., 2015). Harmane alkaloids are also frequently associated with *Passiflora* species, especially *P. incarnata* (Lutomski and Malek, 1975; Lutomski et al., 1975). Additionally, several saponins have been described for this genus, although their occurrence is restricted to certain species (Orsini et al., 1985; Reginatto et al., 2001; Doyama et al., 2005).

As part of our ongoing studies with species of the genus *Passiflora*, we evaluate, in this study, the variability of metabolite presents in the aqueous extracts of four South American *Passiflora* species: *P. alata*, *P. quadrangularis*, *P. bogotensis* and *P. tripartita* var. *mollissima*, focusing specifically on their *C*-glycosylflavonoid and alkaloid composition. The presence of saponins in these species was also evaluated. Chemical profiles were obtained by different analytical methods, such as high performance liquid

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**Table 1**
*Passiflora* species, with their respective local common name, place of collection and identification.

| Species | Local common name | Place of collection/time of year | Identification |
|---|---|---|---|
| *P. quadrangularis* Linn. | Badea, Maracujá-gigante | Rivera, Huila – Colombia (2°59′55″, −75°18′16″)/July 2011 | Prof. Carlos Alberto Parra/Herbarium of the Universidad Nacional de Colombia (COL 572634) |
| *P. alata* Curtis. | Maracujá-doce | Urussanga, Santa Catarina – Brazil (−28°32′9″, −49°18′59″)/January 2011 | Mrs. Ademar Brancher (EPAGRI/Urussanga-SC). Herbarium of the Universidade Federal de Santa Catarina (FLOR 37823) |
| *P. tripartita* var. *mollissima* Holm-Nielsen & Müller Jörgensen | Curuba-de-Castilla | Santa Sofia, Boyacá – Colombia (05°43′01″, −73°36′20″)/June 2011 | Prof. Carlos Alberto Parra/Herbarium of the Universidad Nacional de Colombia (COL 564522) |
| *P. bogotensis* Benth | – | Nemocón, Cundinamarca – Colombia (4°35′60″, −4°4′60″)/May 2011 | Prof. Carlos Alberto Parra/Herbarium of the Universidad Nacional de Colombia (COL 564523) |

chromatography (HPLC), ultra performance liquid chromatography (UPLC) and capillary electrophoresis (CE).

## Materials and methods

### *Chemicals*

Acetonitrile, methanol and formic acid (HPLC-grade) were provided by Tedia® (Rio de Janeiro, Brazil). Water was purified with a Milli-Q system (Millipore®, Bedford, USA). For the CE analysis, stock solutions were prepared from the electrolytes sodium tetraborate (STB) and ammonium acetate ($AcNH_4$) at 100 mmol/l. Sodium hydroxide solution (NaOH) at 1 and 0.1 mol/l were also prepared. All the salts used were of analytical reagent grade, and were provided by Sigma-Aldrich® (St. Louis, USA). All the solvents and solutions were filtered through a 0.22 μm membrane before use. The reference standards orientin, isoorientin, vitexin, isovitexin, vitexin-2″-*O*-rhamnoside, harmol, harmane and harmine (all with purity ≥ 96%) were purchased from Sigma-Aldrich®. Swertisin was previously obtained from *Wilbrandea ebracteata* roots, and identified by NMR spectral data (Santos et al., 1996). The compound 4′-methoxyluteolin-8-*C*-6″-acetylglucopyranoside was previously isolated from *P. tripartita* var. *mollissima* leaves and provided by Prof. Dr. Freddy Ramos (Ramos et al., 2010). Quadranguloside was previously isolated from *Passiflora alata* leaves (Costa et al., 2013).

### *Plant material and preparation of extracts and samples*

Leaves of adult individuals of species of *Passiflora* were collected from different regions of Brazil and Colombia (Table 1). Leaves of the different species were air-dried separately at 40 °C, powdered, and extracted by infusion with boiling water (95 °C, plant:solvent 1:10, w/v) for 10 min. The aqueous extract was then filtered, frozen and lyophilized. The samples for HPLC, UPLC and CE analysis were prepared by dissolving the lyophilized crude aqueous extracts or reference standards in methanol:water (1:1, v/v) and filtering through a 0.22 μm membrane before injection. The concentration of the sample extracts was 1000 μg/ml and for the reference standards, the concentration was 100 μg/ml.

### *HPLC analysis*

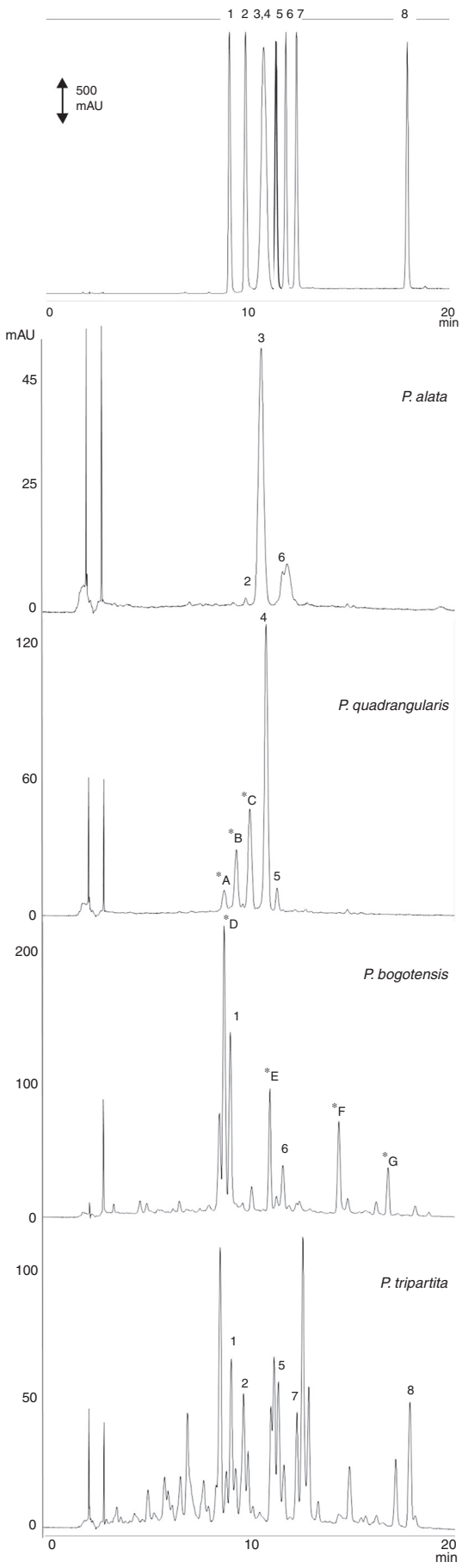
The HPLC analyses were carried out in a PerkinElmer® Series 200 system, equipped with Diode Array Detection (DAD), quaternary pump, on-line degasser and autosampler. The data were processed using the software Chromera® Workstation. The chromatographic analyses for all samples were performed at room temperature (21 ± 2 °C), with an injection volume of 20 μl. The DAD spectra were acquired at the range of 190–450 nm. The peaks in the samples were characterized by comparing the retention time, UV spectra and co-injection with the reference standards. Vertical® VertSep C18 column (250 mm × 4.6 mm i.d.; 5 μm) was used as stationary phase. In the analysis of flavonoids, a gradient system of acetonitrile [solvent A] and formic acid 0.5% [solvent B] was used as mobile phase, in a single step: 15–35% A (0–20 min). The flow rate was kept constant at 1.2 ml/min and the chromatograms were recorded at 340 nm. For alkaloid analysis, the mobile phase used was composed of an aqueous buffer of sodium phosphate dibasic (50 mmol/l, pH 8.0) [A], methanol [B] and acetonitrile [C] at isocratic conditions of 56% A: 12% B: 32% C (0–20 min). The flow rate was kept at 1 ml/min and the UV detection at 245 nm. The chromatographic conditions for the analysis of saponins were previously described by our group (Costa et al., 2013).

### *UPLC analysis*

An UPLC Waters Acquity® H Class system with DAD detector, quaternary pump, on-line degasser and autosampler was used for these analyses. The chromatographic parameters were converted from HPLC to UPLC using the software Empower®. Separations of both flavonoids and alkaloids were carried out in a PerkinElmer® BHE C18 column (100 mm × 2.9 mm i.d.; 1.8 μm). The analyses were also performed at room temperature (21 ± 2 °C), with DAD spectra acquired at the range of 190–450 nm. Flavonoid analysis used a two-steps gradient of acetonitrile [solvent A] and formic acid 0.5% [solvent B]: 15–35% A (0–8 min), followed by 35% A (8–10 min). The flow rate was kept constant at 0.25 ml/min. The injection volume was 3 μl. The chromatogram was recorded at 340 nm. For the alkaloids, the same mobile phase and isocratic system as the HPLC was used, with an analysis time of 7 min. The flow rate was determinate as 0.2 ml/min, with an injection volume of 2 μl. The UV detection was determined at 245 nm.

### *CE analysis*

The analyses were performed on an Agilent 7100 capillary electrophoresis instrument equipped with DAD detector, temperature control device, and autosampler. For all the experiments, a fused-silica capillary (Agilent, model G1600-61232) of 60.5 cm (52 cm effective length), with 50 μm inner diameter and expanded detection window was used. The data were processed using the software Agilent ChemStation®. For the first use, the capillary was pretreated with a pressure flush with 1 mol/l NaOH solution (30 min). Each day, the capillary was conditioned with NaOH 1 mol/l (5 min), waiting time (1 min), Milli-Q water (5 min) and running buffer (5 min). In between runs, the capillary was flushed with running buffer (2 min). The DAD spectra were acquired at the range of 200–500 nm. A method for the analysis of flavonoids was developed using STB (50 mmol/l; pH 9.5, adjusted with NaOH 1 mol/l),




with 20% of MeOH as running buffer. The samples were introduced to the system by hydrodynamic injection (50 mbar/10 s). All separations were performed at a voltage of +25 kV, constant temperature of 30 °C, and direct detection at 390 nm. Apigenin was used as internal standard (I.S.) in order to align the migration time. The alkaloid analysis was performed based on the method previously described by Unger et al. (1997). Briefly, $AcNH_4$ (100 mmol/l; pH 4.0, adjusted with acetic acid), with 50% ACN was used as running buffer. Hydrodynamic injection (50 mbar/5 s) was used, with separation voltage of +15 kV, constant temperature of 15 °C, and detection at 245 nm.

### *Experimental determination of the sensitivity of the methods for alkaloid analysis by UPLC and CE*

The limits of quantification (LOQ) and detection (LOD) for the analytical methods used in the analysis of alkaloids were experimentally determined using standard solutions from the harmane alkaloid, prepared in different concentrations in the matrix (samples), in the range of 50–0.0187 μg/ml. All solutions were prepared and analyzed in triplicate by both techniques. Linearity was determined using nine-point and five-point regression curves, for UPLC and CE, respectively. LOQ was defined by signal:noise ratio of 10:1 and also by relative standard deviation (RSD > 5%). LOD was defined by a signal:noise ratio of 3:1 (ICH, 2005).

## Results and discussion

Plant extracts are often complex mixtures whose therapeutic effect cannot always attributed to a single component, and sometimes, the components responsible for a particular effect are not known. As not all the components have reference standards for identification and quantitation, some quality control analyses may be performed by a fingerprint analysis, in which the experimental data from the chemical analysis of different extracts is compared without accurate quantification. This method is accepted by the World Health Organization for the quality control of herbal medicines (WHO, 1991).

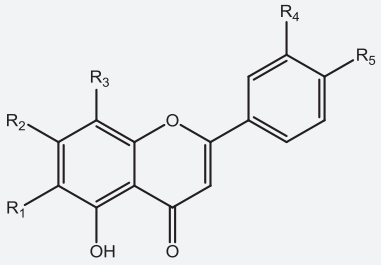
### C-*glycosylflavonoid analysis*

For the development of the chromatographic methods, seven authentic samples of *C*-glycosylflavonoids were used as reference compounds. After evaluating several parameters, including the distinct chromatographic systems described in literature, the initial conditions used were determined as those that allowed good resolution between all reference compounds, and enabled the differentiation of the extracts analyzed, according to the flavonoid profile. The analytical parameters were then optimized, based on visualization of the maximum number of peaks, their resolution index, the time required for the analysis, and the simplicity of the method. The flavonoid fingerprints obtained for each species in the final selected method are presented in Fig. 1, and the peak assignments for each extract are described in Table 2.

Among the four analyzed species, *P. alata* and *P. quadrangularis* presented the least complex flavonoid profile. It is notable

**Fig. 1.** HPLC chromatograms of flavonoids standards (upper chromatogram; normalized view) and aqueous extracts of the leaves of *Passiflora* species. 1: isoorientin, 2: orientin, 3: vitexin-2″-*O*-rhamnoside, 4: vitexin-2″-*O*-xyloside, 5: vitexin, 6: isovitexin, 7: swertisin, and 8: 4′-methoxyluteolin-8-*C*-6″-acetylglucopyranoside. *A: orientin-2″-*O*-glucoside, *B: orientin-2″-*O*-xyloside, *C: vitexin-2″-*O*-glucoside (identified by Gazola, 2014), *D: isoorientin-2″-*O*-rhamnoside, *E: isovitexin-2″-*O*-rhamnoside, *F: luteolin-6-*C*-α-L-rhamnopyranosyl-(1→2)-(6″-*O*-acetyl)-β-D-glucopiranoside, and *G: apigenin-6-*C*-α-L-rhamnopyranosyl-(1→2)-(6″-*O*-acetyl)-β-D-glucopiranoside (identified by Costa et al., 2015). For details of the chromatographic method, see the 'Materials and methods' section.

**Table 2**
Peak assignments of the aqueous extracts by HPLC–DAD.




| Flavonoids | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Rt[a] (min) | UV $\lambda_{max}$ (nm) | Species |
|---|---|---|---|---|---|---|---|---|
| Isoorientin (**1**) | glu | H | H | OH | OH | 9.2 | 258, 268sh, 348 | *P: bogotensis*; *P. tripartita* var. *mollissima* |
| Orientin (**2**) | H | H | glu | OH | OH | 9.8 | 258, 268sh, 348 | *P. alata*; *P. tripartita* var. *mollissima* |
| Vitexin-2″-*O*-rhamnoside (**3**) | H | H | glu-*O*-rham | H | OH | 10.5 | 269, 335 | *P. alata* |
| Vitexin-2″-*O*-xiloside (**4**) | H | H | glu-*O*-xil | H | OH | 10.8 | 269, 335 | *P. quadrangularis* |
| Vitexin (**5**) | H | H | glu | H | OH | 11.4 | 269, 338 | *P. quadrangularis*; *P. tripartita* var. *mollissima* |
| Isovitexin (**6**) | glu | H | H | H | OH | 11.7 | 269, 338 | *P. alata*; *P: bogotensis* |
| Swertisin (**7**) | glu | $OCH_3$ | H | H | OH | 12.3 | 270, 334 | *P. tripartita* var. *mollissima* |
| 4′-Methoxyluteolin-8-*C*-6″-acetylglucopyranoside (**8**) | H | H | glu-*O*-acetyl | OH | $OCH_3$ | 17.8 | 269, 295, 346 | *P. tripartita* var. *mollissima* |

glu, glucose; rham, rhamnose; xil, xiloside.
[a] Peak numbers in Fig. 1.

that the major flavonoids of both species have almost the same retention times. This observation has previously been described by our research group, the two distinct major compounds being identified as vitexin-2″-*O*-rhamnoside (**3**) (*P. alata*) and vitexin-2″-*O*-xyloside (**4**) (*P. quadrangularis*), proposed as chemical markers for these species (Costa et al., 2013). Additionally, orientin (**2**) and isovitexin (**6**) were identified in *P. alata*, while vitexin (**5**) was observed only in *P. quadrangularis*. Other *C*-glycosylflavonoids from *P. quadrangularis*, which have been isolated and identified (Gazola, 2014), were also assigned (Fig. 1, peaks *A–*C). Some of these compounds (orientin-2″-*O*-xyloside, vitexin-2″-*O*-glucoside, vitexin-2″-*O*-xyloside) have also been described by Sakalem and co-workers (2012).

A higher accumulation of flavonoids was observed in the extract of *P. bogotensis*, although only two flavonoids could be identified by spiking experiments with commercial standards (isoorientin (**1**) and isovitexin (**6**)). Recently, work on the flavonoid composition of *P. bogotensis* leaves has reported the presence of these compounds, together with other *C*-glycosylflavonoids, indicated in Fig. 1 (peaks *D–*G) (Costa et al., 2015).

Among the extracts analyzed, *P. tripartita* var *mollissima* displayed the most complex flavonoid profile. Isoorientin, orientin, vitexin, swertisin and 4′-methoxyluteolin-8-*C*-6″-acetylglucopyranoside could be identified by co-injection and UV spectra. In a previous work, the same authors have evaluated the *C*-glycosylflavonoid profile of *P. tripartita* var. *mollissima* leaves and pericarp by a distinct HPLC method (Zucolotto et al., 2012). Although both are suitable for their purposes, the method described herein allows the great diversity of flavonoids to be better observed, with an analysis time of only 20 min. Simirgiotis and co-workers (2013) have studied the peel and fruit juice of this species, which although different parts of the plant, presented several flavonoids that were structurally related to those observed in our work.

The parameters developed for the HPLC analysis of the reference compounds (authentic samples of *C*-glycosylflavonoids) and the extracts (Fig. 1), were subsequently used in the UPLC analysis, yielding similar results (Fig. 2). Although the HPLC analysis allowed rapid analysis with an adequate resolution between peaks, comparatively, the analysis time by UPLC was reduced by 50% (10 min), with virtually unchanged fingerprints. As for the HPLC analysis, the UPLC analysis also enabled us to differentiate the four extracts, especially in the difficult distinction of the mayor flavonoids from *P. alata* and *P. quadrangularis*.

In addition to the analysis by chromatographic methods, the flavonoid composition was also analyzed by capillary electrophoretic (CE). The literature mostly reports the use of borate buffers as running electrolytes in the analysis of flavonoids by CE (Molnár-Perl and Füzfai, 2005; Marchart et al., 2003; Rijke et al., 2006). Considering that the previously chromatographic analyses have revealed a higher complexity of flavonoids in *P. tripartita* var. *mollissima*, the CE method was initially developed for this extract. Some parameters were evaluated for the change in electrosmotic flow, such as the electrolyte concentration, voltage, capillary temperature and injection volume. The conditions that provide the best separation for *P. tripartita* var. *mollissima* (see 'Materials and methods' section) were applied to the other extracts (Fig. 3), enabling to distinguish the flavonoid fingerprints for each species.

Compared with the chromatographic techniques, the profiles obtained by CE showed also a short time analysis, good peak resolution and symmetry. Qualitatively, another substantial difference between the chromatographic and electrophoretic methods is the detection wavelength. In HPLC, flavonoids are usually detected in the range of 330–350 nm, which is the band of maximum absorption for these compounds, providing selectivity to the method. However, the detection in CE was performed at 390 nm, due to the borate-flavonoid complex at pH 9.5, which leads to a bathochromic effect for this band.

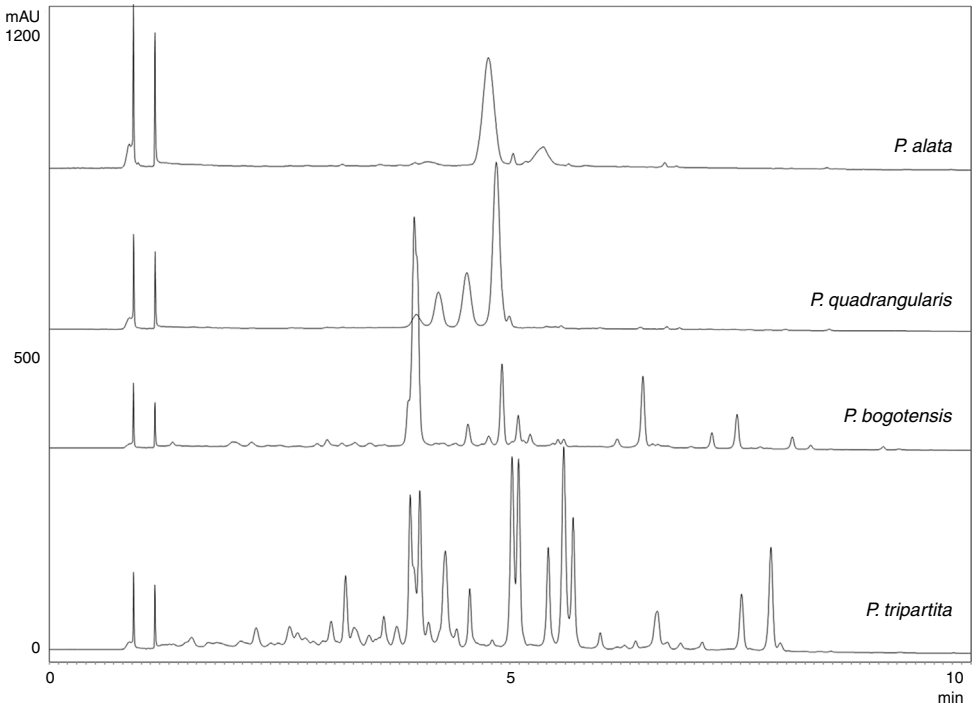



**Fig. 2.** UPLC chromatogram of flavonoids from aqueous extracts of the leaves of *Passiflora* species. For details of the chromatographic method, see the 'Experimental' section.

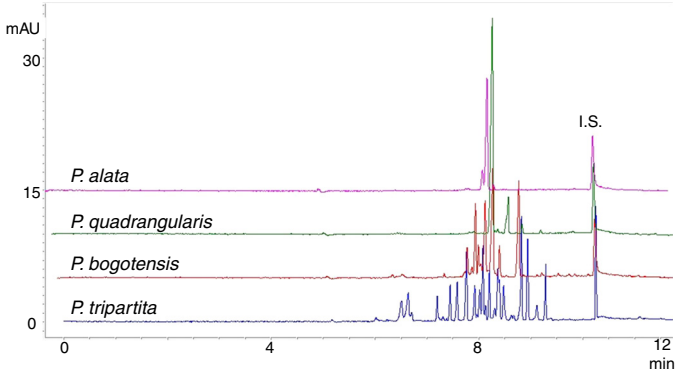



**Fig. 3.** CE electropherogram of flavonoids from aqueous extracts of the leaves of *Passiflora* species. Internal standard (I.S.): apigenin. For details of the capillary electrophoretic method, see the 'Materials and methods' section.

### *Alkaloids analysis*

Some previous studies report the presence of alkaloids in *P. incarnata* and *P. edulis* (Poethke et al., 1970; Lutomski et al., 1975). Nevertheless, more recent works, with more sensitive methods, have questioned the presence or levels of these compounds (Speroni and Minghetti, 1988; Rehwald et al., 1995; Grice et al., 2001).

In the alkaloid analysis presented in this study, chromatographic and electrophoretic methods were developed using the standards harmol, harmane and harmine, compounds that have previously been described for *P. incarnata* and *P. edulis* (Poethke et al., 1970; Lutomski et al., 1975). Qualitatively, the presence of these alkaloids was not observed in the crude aqueous extract from the leaves of these species by HPLC, UPLC or CE (Fig. 4). For this reason, a calibration curve for harmane was built in UPLC and CE (the techniques with the fastest methods) to determine the linearity and sensitivity of these methods. The results are presented in Table 3.

The quantitative data showed an excellent linear relationship between peak area and concentration ($r^2 > 0.999$) for both techniques. About the sensitivity of these methodologies, it was observed that UPLC presented a sensitivity 15-fold higher then CE in these conditions, which can be mainly explained by the difference of the interparticules spaces in the UPLC column and the internal diameter of the capillary column in CE.

Considering the determined limits for these methods, it can be stated that the aqueous extracts analyzed in this work do not have harmane type alkaloids at levels exceeding 0.0187 μg/ml (=0.0187 ppm). This result is in accordance with some previous quantitative works by HPLC, which also did not detect alkaloids in the *Passiflora* species investigated, at concentrations higher than 0.1 ppm (Rehwald et al., 1995). Grice and co-workers (2001) detected alkaloids in commercial samples of *P. incarnata* at levels lowers than 0.018 ppm, but using a fluorescence detector instead of a diode array detector.

Nevertheless, the non-detection of alkaloids, despite appearing as a negative result, has great relevance. The aqueous extracts evaluated were prepared according to the use of *Passiflora* leaves in folk medicine. Thus, it was demonstrated that these compounds are absent in the traditional preparations. However, these data do not rule out the presence of harmane alkaloids in these species,

**Table 3**
Calibration data of harmane.

| Technique | Linearity range (μg/ml) | Calibration equation[a] | Correlation factor ($r^2$) | LOQ[b] (μg/ml) | LOD[b] (μg/ml) |
|---|---|---|---|---|---|
| CE | 50.0–1.0 | $y = 2.4144x + 1.0596$ | 0.9997 | 0.5 | 0.25 |
| UPLC | 50.0–0.065 | $y = 123720x - 6479.9$ | 0.9999 | 0.0315 | 0.0187 |

[a] CE, five points ($n = 3$); UPLC, nine points ($n = 3$).
[b] LOQ, limit of quantification; LOD, limit of detection.

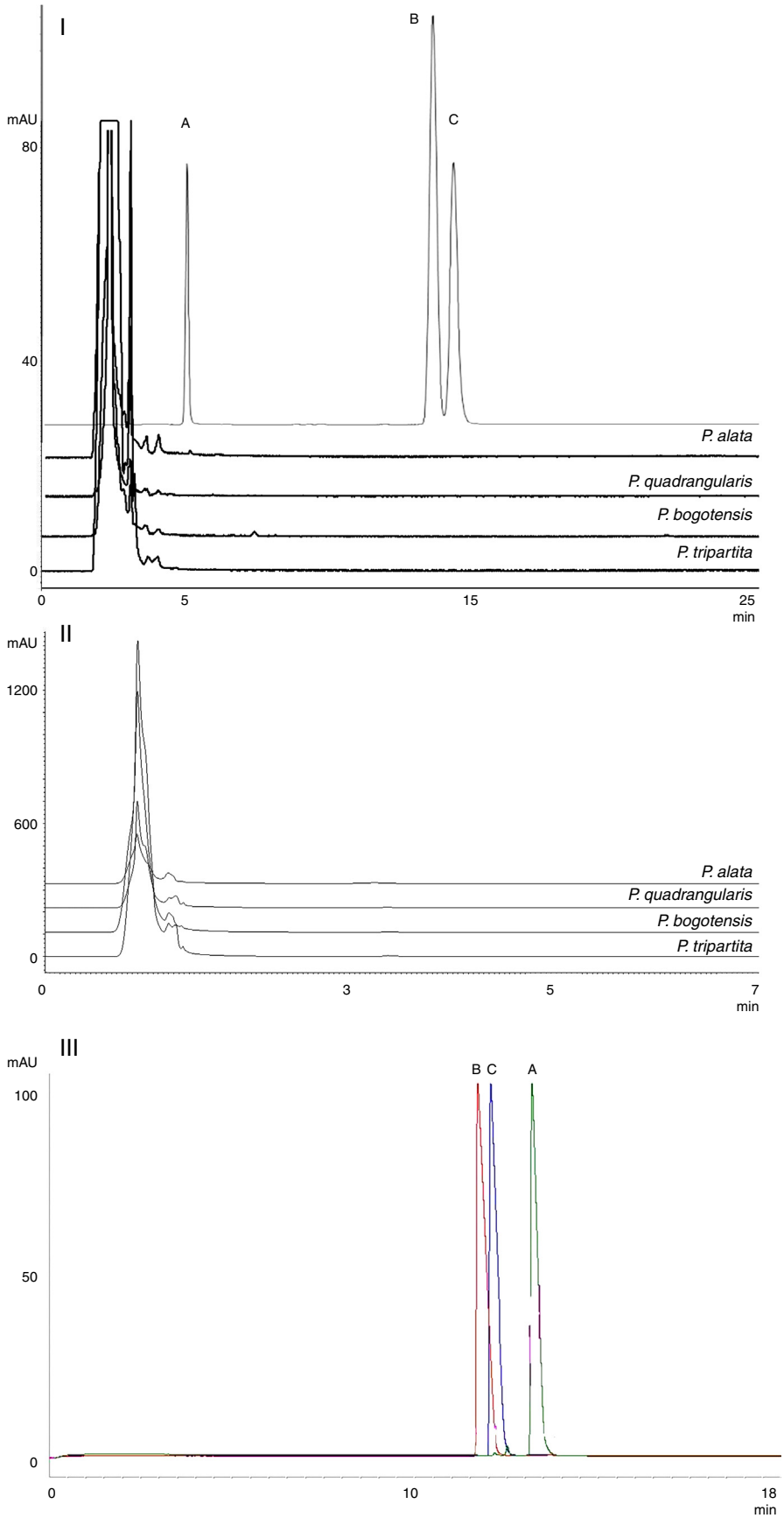



**Fig. 4.** Chromatograms (I: HPLC; II: UPLC) and electropherogram (III) of alkaloids standards (A: harmol; B: harmane; C: harmine) and of aqueous extracts of the leaves of *Passiflora* species. For details of the methods, see the 'Experimental' section.

which may be present in other organs of the plant, or even in the leaves, in which a specific alkaloid extractive method could be used for this purpose.

*Saponin analysis*

Even though many saponins have been described for the genus *Passiflora*, the occurrence of these metabolites is restricted to just a few species. The presence of saponins in *P. alata* and *P. quadrangularis* has already been reported in previous works of our group (Reginatto et al., 2004; Birk et al., 2005), as has a comparative analysis by HPLC of these two species (Costa et al., 2013). The additional results presented herein, using the same chromatographic conditions, indicate that the extracts of *P. bogotensis* and *P. tripartita* var. *mollissima* leaves showed no evidence of these compounds (see supplementary material).

In conclusion, fast and simple analytical methods for the fingerprinting of flavonoids and alkaloids from *P. alata*, *P. quadrangularis*, *P. bogotensis* and *P. tripartita* var. *mollissima* extracts were successfully established by three different techniques, showing good resolution and sensitivity. A wide diversity of flavonoids was observed for these four species, while saponins were accumulated only in *P. alata* and *P. quadrangularis* extracts. Alkaloids, whose presence is controversial in previous papers, were not detected by any of the methods used. The analytical methods and techniques reported herein are suitable for quality control analysis of these metabolites in plant samples, and would be of great help in future works with other *Passiflora* species.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Confidentiality of data.** The authors declare that they have followed the protocols of their work center on the publication of patient data.

**Right to privacy and informed consent.** The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

## Authors' contributions

GMC performed the extraction, analytical work, data analysis, and drafting of the paper. ACG contributed to the chromatographic analysis. SMZ contributed to the plant collection and extraction. LC and FAR contributed to the collection of the plants, preparation of the voucher specimen, supervision of laboratory work, and critical reading of the manuscript. FHR and EPS designed the study, supervised the laboratory work, and contributed to a critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

## Conflicts of interest

The authors declare no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.02.005.

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